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Cell Interactions in the Induction of Tolerance: The Role of Thymic Lymphocytes

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Summary. Thymectomized, lethally irradiated, bone marrow reconstituted mice were treated with a large dose of sheep red blood cells (SRBC) over the course of 30 days. They were unable to respond to further antigenic challenge for one month. Fifteen million thymocytes given 4 days after the termination of treatment restored their ability to respond.

The same antigenic treatment given to similar chimeras, which differed only in having had 15×10^6 thymus cells added to the bone marrow inoculum, also abolished the response to further antigenic challenge. In contrast to chimeras without thymus cells present during the course of treatment, the later addition of thymocytes to these animals did not restore their response. It did, however, restore the response to a second challenge of antigen given 17 days after the addition of thymocytes. This response was the same as non-treated animals given only one injection of thymocytes and significantly less than non-treated animals given thymocytes twice.

The following explanation of these results is offered. Bone marrow derived (BMD) lymphocytes that can make antibody without assistance of thymus derived (TD) lymphocytes were made tolerant in the absence of TD cells. Thymus dependent BMD cells were not. New cells, coming from the bone marrow, broke the tolerant state within a month.

When TD cells were present both populations of BMD cells, as well as the TD cells, were made tolerant. New BMD cells regenerating from the bone marrow abrogated the tolerant state of the BMD population. This breaking of tolerance could only be seen in mice given additional thymocytes as the tolerance of the TD cells was not broken in the absence of a thymus.

Thus, the induction of tolerance as well as the induction of immunity in thymus dependent BMD cell populations, seems to require the co-operation of TD cells.

INTRODUCTION

Recent work has established that interactions between two types of lymphocytes play an important role in the production of antibodies to heterologous red blood cells in the mouse (Claman, Chaperon and Triplett, 1966; Davies, Leuchars, Wallis, Marchant and Elliott, 1967; Mitchell and Miller, 1968a). These lymphocytes are distinguished by the fact that they enter the peripheral pool of cells from different source organs. Those that enter via the thymus [referred to as thymus-derived (TD) cells] have been shown to respond

to antigenic stimulation by mitosis and protein synthesis (Davies, Leuchars, Wallis and Koller, 1966). They do not, however, release significant amounts of circulating antibody (Claman *et al.*, 1966; Davies *et al.*, 1967; Mitchell and Miller, 1968a).

It is not yet clear whether the other lymphocytes in the response, referred to as bone marrow-derived (BMD) cells* come directly from the bone marrow or pass through another source organ, such as an equivalent to the bursa of Fabricius of avian species (Cooper, Gabrielson and Good, 1967).

It is clear, however, that these are the cells that produce antibody in the response to sheep red blood cells (SRBC) (Davies *et al.*, 1967; Mitchell and Miller, 1968a, b; Davies, Leuchars, Wallis, Sinclair and Elliott, 1968; Miller and Mitchell, 1968; Nossal, Cunningham, Mitchell and Miller, 1968). Although it appears that a few BMD cells (mostly 19S producers) can make antibody without the assistance of TD cells, most require TD cell help, particularly in the primary response (Davies *et al.*, 1967, 1968).

At present little is known about the role these cells may have in the induction of tolerance. Several investigators have presented evidence which suggests the TD cell may be made tolerant (Isakovic, Smith and Waksman, 1965; Gershon, Wallis, Davies and Leuchars, 1968; Taylor, 1968; Abdou and McKenna, 1968) but it has not yet been established whether the BMD cell may also be. The experiments reported below test this possibility by determining whether pretreatment with antigen can abolish the ability of the BMD cell to co-operate with normal thymocytes. In addition they test what role co-operation of TD cells might play in this event.

EXPERIMENTAL PLAN

The general outline of the experimental plan is presented in Fig. 1. Each group studied has been given a number which is referred to when they are discussed in the text.

The role of the TD cell in the production of tolerance was tested by heavily pretreating two groups of mice; one deprived of TD cells and one with TD cells present. Uninoculated animals in both groups served as controls.

To obtain mice without TD cells, adult CBA mice were thymectomized at 7–8 weeks of age. One week later they were lethally irradiated and given 5×10^6 syngeneic bone marrow cells, intravenously.

To obtain mice with TD cells the same procedure was carried out but 15×10^6 thymocytes were added to the bone marrow inoculum. This is a convenient number of cells to use for several reasons.

(1) It is large enough to give a measureable and repeatable effect in reconstituting the response to SRBC.

(2) It is a small enough number to assure that contamination of the inoculum with significant numbers of non-thymic lymphocytes is minimal.

(3) It is small enough to allow the addition of a second inoculation of thymocytes to significantly augment the response to SRBC.

(4) Because of its small size any effect it might produce should be quite significant. (The corollary to this, of course, is that the absence of an effect is less significant.)

* They are so called because, in radiation chimeras restored with bone marrow and thymus cells, these cells come from the bone marrow inoculum. It is worth emphasizing that the lymphocytes within the thymus most probably also had their early origins in the bone marrow (Micklem, Ford, Evans and Gray, 1966; Owen and Ritter, 1969).

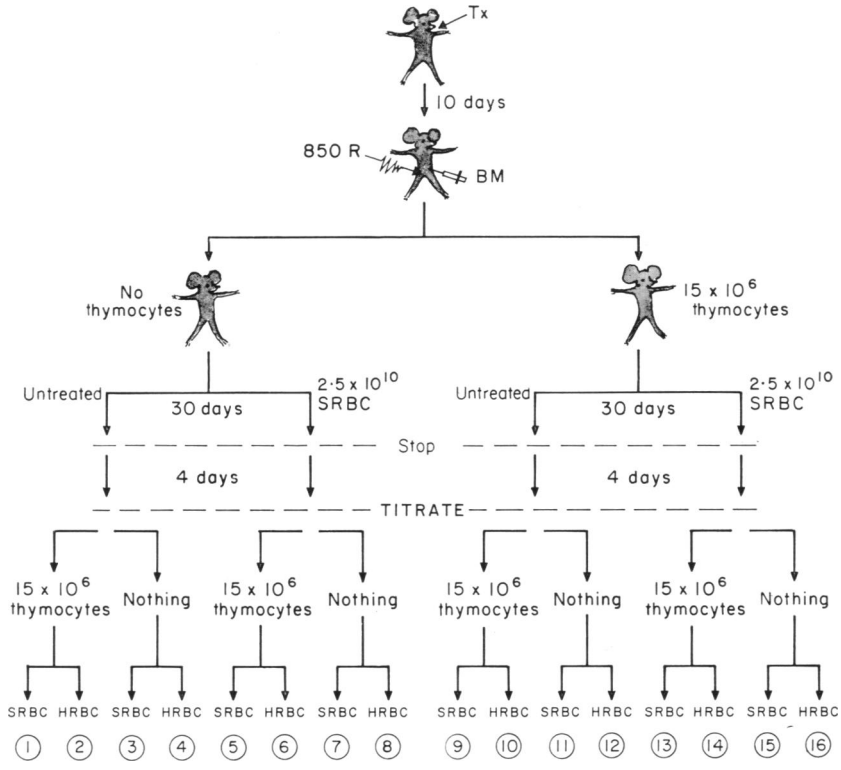

FIG. 1. Plan of experiments. The figures in circles indicate the experimental group number and these numbers are referred to when the groups are discussed in the text.

Immediately after inoculation and on the next 2 days, half of each group was given 3×10^9 SRBC intraperitoneally. They were then given 4×10^9 SRBC/week for 4 weeks (in four weekly injections) making a total dose of approximately $2{\cdot}5\times10^{10}$ SRBC. The remaining mice were not injected with SRBC. Four days after the last injection, serum was collected from all mice and titrated against SRBC and horse red blood cells (HRBC). Half of each of the four groups were then given 15×10^6 normal thymocytes intravenously. The resultant eight groups were then immunized; half of each group with 5×10^8 SRBC and half with 5×10^8 HRBC as a specificity control. Thus, sixteen groups of mice were produced.

The sera of these mice were then titrated for haemagglutinating antibodies against SRBC and HRBC on days 5, 7, 10 and 15 after immunization. A second injection of the homologous immunizing antigen was given on day 17.

A few experiments were done that deviated somewhat from the general outline and these are described in the text.

MATERIALS AND METHODS

Mice

Male CBA mice were used in these experiments. They were either strain CBA/H from the Chester Beatty colony or strain CBA/J from Jackson Laboratories, Bar Harbor, Maine.

Thymectomy

Thymectomies were performed on adult mice, 7–8 weeks of age, under light ether anaesthesia following the technique of Miller (1960). At the termination of experiments all mice were autopsied and thymic remnants were searched for. None were found in any animals used in these experiments.

Irradiation

Two different X-ray machines were used; a Westinghouse 220 kV or a Siemans 250 kV. A total of 850 R was delivered in all cases, at dose rates of 60 or 85 R/min.

Cell suspensions

Bone marrow cell suspensions were prepared by washing out the femurs of adult syngeneic mice with cold sterile tissue culture medium 199. Thymus cell suspensions were prepared by gently teasing thymuses of syngeneic weanling (4–5 weeks of age) mice between sterile glass slides in cold medium 199. They were filtered through gauze and washed before injection. Counts of viable cells were made in a hemocytometer using the Trypan blue dye exclusion method. The cells were inoculated, as detailed above, intravenously via the tail vein.

Red blood cells

These were obtained in Alsevers solution washed three times before use and inoculated intraperitoneally in a final volume of 0·2 ml as detailed above.

Bleeding

Bleeding was done with capillary pipettes placed in the retro-orbital sinus. Serum was separated and used for titration within 24 hours. Individual mice were ear-marked so that each could be followed serially.

Titrations

Sera were individually titrated by the microhaemagglutination technique described by Sever (1962). The titres were expressed as the $\log_2$ of the last well showing macroscopic agglutination. Thus, if the undiluted serum showed no agglutination the titre is expressed as $\bar{1}$. A titre of 0 means agglutination occurred with whole serum but not at a 1 : 2 dilution in isotonic saline. After the results had been recorded (all results were read separately by two observers in a 'blind' fashion) the red cells were resuspended by gentle tapping of the plates and 0·025 ml of 0·1 M 2-mercaptoethanol (ME) was added to each well. The cells were allowed to resettle at room temperature and end-points were read as before. These titres were taken to represent ME resistant (MER) antibody. This method of ME inactivation has been studied at some length and has been shown to produce the same results as more standard techniques (Scott and Gershon, 1970). It was used in these studies in order to minimize the blood loss of experimental animals. MER antibody may be considered roughly equivalent to 7S antibody under ordinary circumstances (Adler, 1965). MER titres are reported below only when significant differences between test and control animals were present.

Selection of animals

The results reported below are from animals that had no antibody present in their sera

on the day of immunization. About 50 per cent of pretreated animals and 2 per cent of non-pretreated animals were thus eliminated from this study. Because of this elimination, pretreated groups were somewhat smaller in number than non-pretreated ones, but no group in any individual experiment was comprised of less than five mice.

Statistical analysis

Student's *t*-test was the method used in all statistical analyses.

RESULTS

I. THE EFFECT OF ANTIGEN PRETREATMENT IN THE ABSENCE OF THYMOCYTES (Groups 1–8 in Fig. 1)

(A) Without the addition of thymocytes after pretreatment (the thymus independent response) (Groups 3, 4, 7 and 8 in Fig. 1)

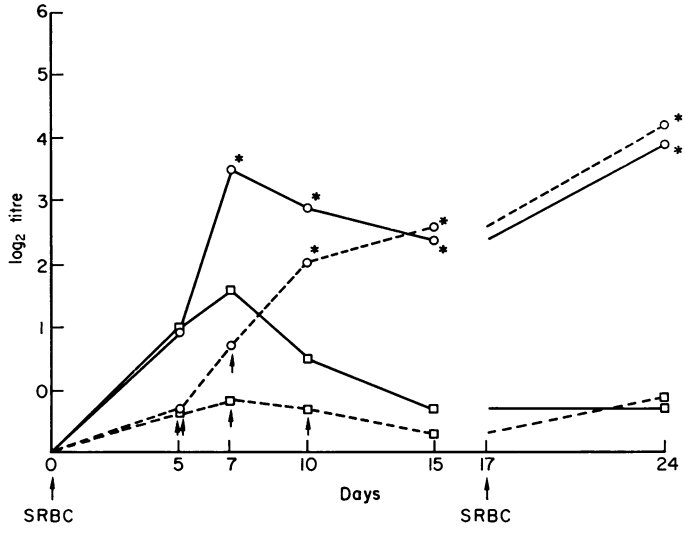

FIG. 2. Effect of pretreating animals *lacking* thymus derived cells with $2{\cdot}5 \times 10^{10}$ sheep red blood cells on the subsequent immune response to 5×10^{8} sheep cells. ○, Animals given 15×10^{6} thymocytes after pretreatment; □, animals not given thymocytes after pretreatment ;———; animals not pretreated with antigen; - - - ; animals pretreated with $2{\cdot}5 \times 10^{10}$ sheep red blood cells before antigenic challenge. ○—○, Group 1; □—□, Group 3; ○ - - ○, Group 5; □ - - □, Group 7. Group numbers refer to the experimental groups in Fig. 1. Asterisks indicate a statistically significant increase in antibody produced by the addition of thymocytes (solid *versus* solid lines; broken *versus* broken lines). Arrows indicate a statistically significant reduction in antibody produced by pretreatment with sheep cells (circles *versus* circles; squares *versus* squares).

Fig. 2 shows that animals pretreated with SRBC (Group 7) did not respond to an immunizing injection of antigen. The non-pretreated controls (Group 3) made a small, transient response of 2-mercaptoethanol sensitive (MES) antibody. The difference between these two groups was statistically significant on days 5, 7 and 10 ($P<0{\cdot}02$, 0·001 and 0·01,

respectively). Neither group responded to a second injection of antigen on day 17 (both groups responded to a third injection of antigen given 1 month after the second injection, in a typical primary fashion).

These results were confirmed in another experiment where the mice were kept 90 days before a second challenge, at which time both groups were able to respond (Groups 3 and 7 in Fig. 3) in a fashion that might suggest a secondary response. However, since no uninoculated controls were done, this point cannot be substantiated.

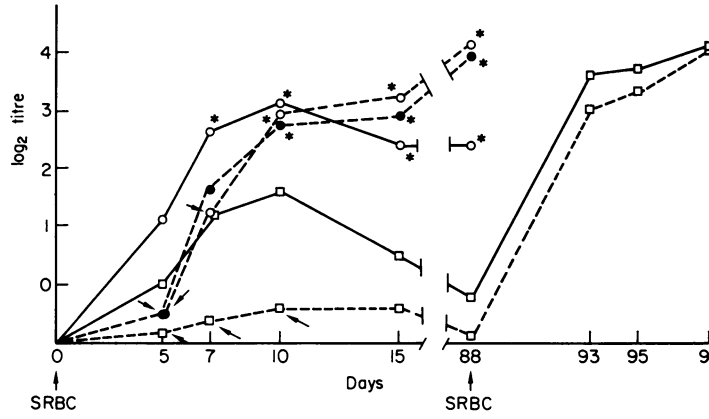

FIG. 3. Effect of pretreating animals *lacking* thymus derived cells with $2{\cdot}5 \times 10^{10}$ sheep red blood cells on the subsequent immune response to 5×10^{8} sheep cells. ○, Animals given 15×10^{6} thymocytes after pretreatment; ●, animals given 15×10^{6} thymocytes with sheep cells 3 days after the animals with open circles; □, animals not given thymocytes after pretreatment; ———, animals not pretreated with antigen; - - -, animals pretreated with $2{\cdot}5 \times 10^{10}$ sheep red blood cells before antigenic challenge. ○—○, Group 1; □—□, Group 3; ○ -- ○, Group 5; ●--●, Group 5 (thymocytes delayed); □--□, Group 7. Group numbers refer to the experimental groups in Fig. 1. Asterisks indicate a statistically significant increase in antibody produced by the addition of thymocytes (solid *versus* solid lines; broken *versus* broken lines). Arrows indicate a statistically significant reduction in antibody produced by pretreatment with sheep cells (circles *versus* circles; squares *versus* squares).

The specificity of the suppression, as can be seen in Fig. 4 was poor. Animals pretreated with SRBC (Group 8), except for day 5, made significantly less antibody in response to immunization with HRBC than did non-pretreated controls (Group 4) ($P<0{\cdot}01$ on days 7 and 10). As above, neither group responded to a second challenge on day 17.

In a second experiment a similar cross suppression was observed, again with the exception of the earliest response.

The possible significance of this apparent lack of specificity is discussed below.

Comment. These results suggest that antibody making cells which do not require the assistance of T.D. cells to make antibody to SRBC may be paralysed by antigenic overloading, and that T.D. cell co-operation is not required for this event to occur.

(*B*) *With the addition of thymocytes after pretreatment* (the thymus dependent response) (Groups 1, 2, 5 and 6 in Fig. 1)

It can be seen in Fig. 2 that the addition of 15×10^{6} thymocytes to pretreated animals (Group 5) did not significantly augment their immune response to SRBC on days 5 and 7 after challenge (compare with Group 7). However, by day 10 ($P<0{\cdot}01$) and thereafter ($P<0{\cdot}001$) a significantly increased antibody titre was produced as a result of the thymic

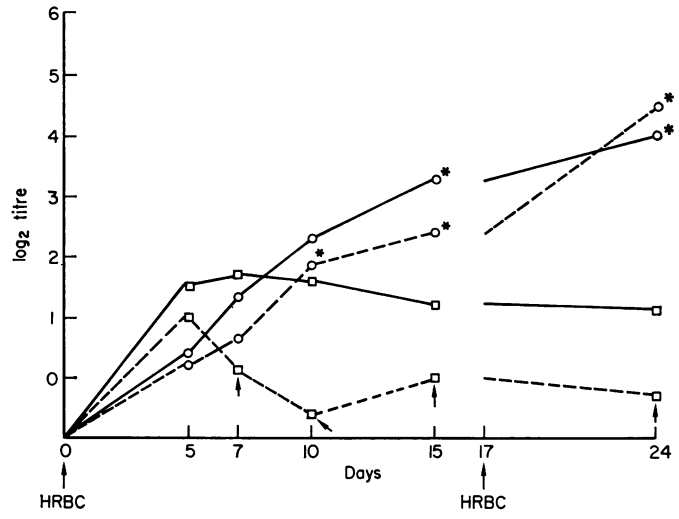

FIG. 4. Effect of pretreating animals *lacking* thymus derived cells with $2{\cdot}5 \times 10^{10}$ sheep red blood cells on the subsequent immune response to 5×10^{8} horse red blood cells. ○, Animals given 15×10^{6} thymocytes after pretreatment; □, animals not given thymocytes after pretreatment; ———, animals not pretreated with antigen; ....., animals pretreated with $2{\cdot}5 \times 10^{10}$ sheep red blood cells before antigenic challenge. ○—○, Group 2; □—□, Group 4; ○--○, Group 6; □--□, Group 8. Group numbers refer to the experimental groups in Fig. 1. Asterisks indicate a statistically significant increase in antibody produced by the addition of thymocytes (solid *versus* solid lines; broken *versus* broken lines). Arrows indicate a statistically significant reduction in antibody produced by pretreatment with sheep cells (circles *versus* circles; squares *versus* squares).

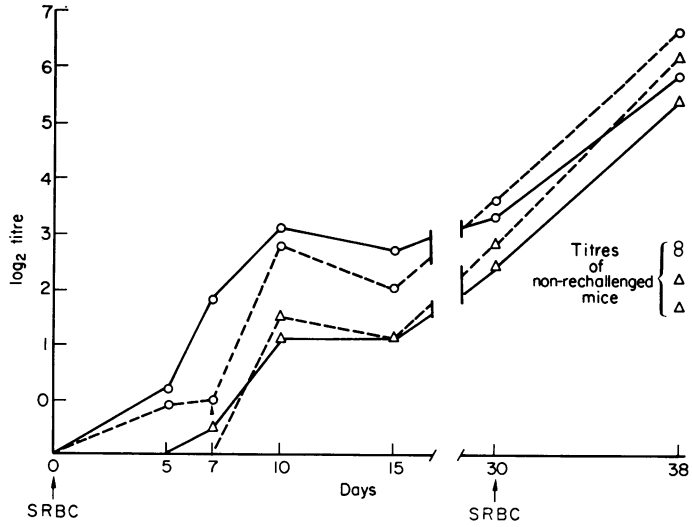

FIG. 5. Effect of pretreating animals *lacking* thymus derived cells with $2{\cdot}5 \times 10^{10}$ sheep red blood cells on the subsequent immune response to 5×10^{8} sheep red blood cells. All animals were given 15×10^{6} thymocytes after pretreatment. ○, Total antibody; △, antibody after treatment with 2-mercaptoethanol; ———, non-pretreated animals; - - -, pretreated animals. ○—○, Group 1; △—△, Group 1 (MER ab); ○--○, Group 5; △--△, Group 5 (MER ab). Group numbers refer to the experimental groups in Fig. 1. Arrows indicate a statistically significant reduction in antibody produced by pretreatment with sheep cells (circles *versus* circles; triangles *versus* triangles).

cell inoculation. By comparing these animals with non-pretreated controls (Group 1) it can be seen that their antibody titre was depressed on day 5 ($P<0{\cdot}05$) and day 7 ($P<0{\cdot}01$). From day 10 onward (up to 100 days) the two groups had similar titres. These results were confirmed in two separate experiments with significant suppression of the antibody response early and normal titres from day 10 onward. At no time in any of these three experiments were the MER antibody titres of the two groups significantly different. The results of one of these experiments is presented in Fig. 5.

One further experiment was performed with these groups. An additional three days was allowed to elapse between the termination of the pretreatment and the addition of thymocytes, to see if the recovery noted above on day 10 could be foreshortened. As can be seen in Fig. 3 (Groups 1, 5 and 5 TD cells delayed) no significant recovery took place in the absence of thymocytes.

Specificity in this instance was quite good. No significant depression of antibody formation to HRBC was produced by pretreatment with SRBC (similarly in Groups 2 and 6 Fig. 4). Repeat of this experiment on two occasions produced similar results except for a precocius response on day 5 in pretreated animals on one occasion (Fig. 6). Since this finding was not repeatable its significance is unknown. It is clear, however, that no suppression of the HRBC response was produced by pretreatment with SRBC in these animals.

Comment. SRBC pretreatment of mice in the absence of thymocytes does not impair subsequent co-operation between their MER antibody-making precursor cells and normal thymocytes. It does, however, temporarily diminish the response of MES-antibody making cells even after the addition of thymocytes. The significance of this point is considered in the discussion.

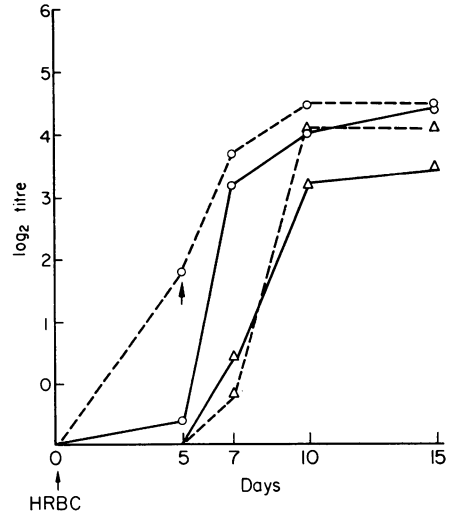

FIG. 6. Effect of pretreating animals *lacking* thymus derived cells with $2{\cdot}5 \times 10^{10}$ sheep red blood cells on the subsequent immune response to 5×10^{8} horse red blood cells. All animals were given 15×10^{6} thymocytes after pretreatment. ○, Total antibody; △, antibody after treatment with 2-mercaptoethanol; ———, non-pretreated animals; - - -, pretreated animals. ○—○, Group 2; △—△, Group 2 (MER ab); ○--○, Group 6; △--△, Group 6 (MER ab). Group numbers refer to the experimental groups in Fig. 1. Arrows indicate a statistically significant increase in antibody produced by pretreatment with sheep cells (circles *versus* circles; triangles *versus* triangles).

II. THE EFFECT OF ANTIGEN PRETREATMENT IN THE PRESENCE OF THYMOCYTES (Groups 9–16 in Fig. 1).

(*A*) *Without the addition of thymocytes after pretreatment* (Groups 11, 12, 15 and 16)

No significant immune response either primary or secondary, occurred in pretreated animals (Fig. 7, Group 15). On the other hand, non-pretreated controls (Group 11) responded in the same fashion as animals that received a single inoculation of 15×10^6 thymocytes on day −34 (Group 1 Fig. 2) instead of on day 0. The depression of the antibody response produced by the pretreatment was statistically significant on day 7 and thereafter, when compared with non-pretreated controls (Group 15 *versus* 11).

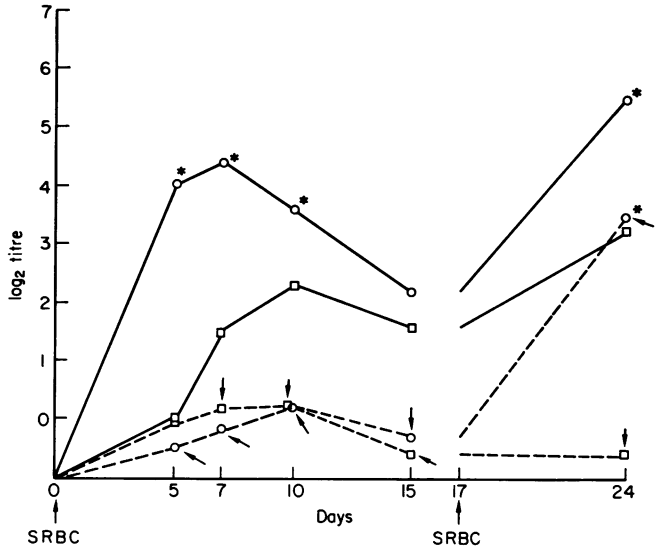

FIG. 7. Effect of pretreating animals *in the presence* of thymus derived cells with $2{\cdot}5 \times 10^{10}$ sheep red blood cells on the subsequent immune response to 5×10^8 sheep cells. ○, Animals given 15×10^6 thymocytes after pretreatment; □, animals not given thymocytes after pretreatment; ———, animals not pretreated with antigen; - - -, animals pretreated with $2{\cdot}5 \times 10^{10}$ sheep red blood cells before antigenic challenge. ○—○, Group 9; □—□, Group 11; ○--○, Group 13; □--□, Group 15. Group numbers refer to the experimental groups in Fig. 1. Asterisks indicate a statistically significant increase in antibody produced by the addition of thymocytes (solid *versus* solid lines; broken *versus* broken lines). Arrows indicate a statistically significant reduction in antibody produced by pretreatment with sheep cells (circles *versus* circles; squares *versus* squares).

The specificity of this depression was poor in the primary but improved in the secondary response. In Fig. 8 it can be seen that animals pretreated with SRBC and given HRBC without thymocytes (Group 16) made a very poor response that was depressed on all days of the primary response when compared with non-pretreated controls (Group 12). However, 7 days after a second immunization they made a significant response, although it was deficient in MER antibodies ($\log_2$ titre: control 3·9; test group 1·4, $P<0{\cdot}01$). Although the tertiary and subsequent responses are not reported in this paper it is of note that a relatively deficient HRBC response remained for a long time.

Comment. The SRBC pretreatment of mice with thymocytes present, similar to its effect in mice deprived of thymocytes, leads to a state of unresponsiveness to further challenge. This paralysis lasts for more than 3 weeks.

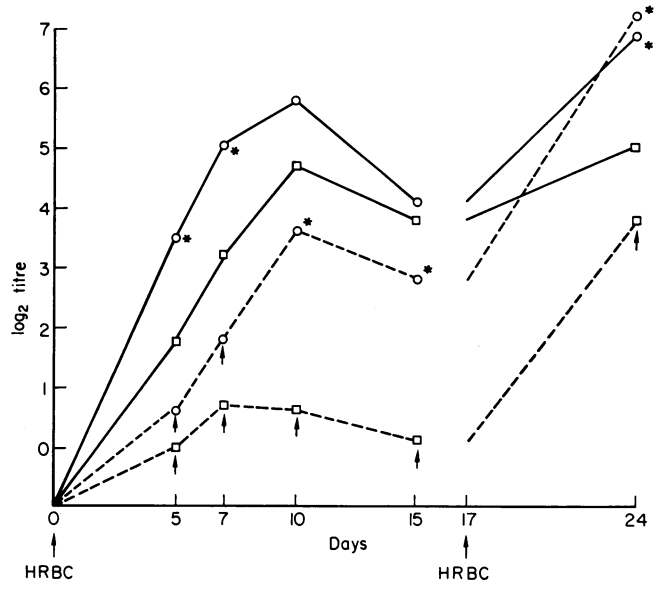

FIG. 8. Effect of pretreating animals *in the presence of* thymus derived cells with $2{\cdot}5 \times 10^{10}$ sheep red blood cells on the subsequent immune response to 5×10^{8} horse red blood cells. ○. Animals given 15×10^{6} thymocytes after pretreatment; □, animals not given thymocytes after pretreatment; ———, animals not pretreated with antigen; - - -, animals pretreated with $2{\cdot}5 \times 10^{10}$ sheep red blood cells before antigenic challenge. ○—○, Group 10; □—□, Group 12; ○--○, Group 14; □--□, Group 16. Group numbers refer to the experimental groups in Fig. 1. Asterisks indicate a statistically significant increase in antibody produced by the addition of thymocytes (solid *versus* solid lines; broken *versus* broken lines). Arrows indicate a statistically significant reduction in antibody produced by pretreatment with sheep cells (circles *versus* circles; squares *versus* squares).

(*B*) *With the addition of thymocytes after pretreatment* (Groups 9, 10, 13 and 14)

In giving a second injection of thymocytes to determine if an animal is tolerant, it is important to know if these cells can affect a non-pretreated control. It can be seen in Figs. 7 and 8 that the second inoculation of thymocytes produced a significant increase in antibody production in both the SRBC (Group 9 *versus* 11 in Fig. 7) and the HRBC (Group 10 *versus* 12 in Fig. 8) systems. This was true in both the primary and secondary response ($P<0{\cdot}02$ in both cases).

Although the thymocytes were able to boost non-pretreated controls, they were without effect in the pretreated animals (Group 13 *versus* Group 15 in Fig. 7). This was in sharp contrast with the results presented above in animals pretreated in the absence of thymocytes. The difference between the non-pretreated controls and test animals in this case was highly significant ($P<0{\cdot}001$) on all days of the primary response. Reimmunization of pretreated mice on day 17 resulted however, in a response that was significantly greater ($P<0{\cdot}001$) than that made by animals which had received the same pretreatment but had not been given a second inoculation of thymocytes (Group 13 *versus* Group 15). Although these animals responded to a second immunization, their response was significantly less than that made by non-pretreated controls (Group 9, $P<0{\cdot}02$). It was in fact almost exactly the same as the response of non-pretreated animals which had received only a single inoculation of thymocytes, either on day -34 (Group 11) or on day 0 (Group 1 Fig. 2). MER-antibody titres of these three groups were likewise similar.

The response to HRBC of pretreated animals was also somewhat depressed compared to non-pretreated controls (Groups 14 *versus* 10 Fig. 8). This depression was statistically significant on days 5 ($P<0{\cdot}001$) and 7 ($P<0{\cdot}01$) but on days 10 and 14, although the response remained suppressed, the difference was no longer significant. The response of the two groups was very similar in the secondary response.

Comment. The SRBC pretreatment of mice with thymocytes present, in contrast to its effect in mice deprived of thymocytes, prevents the addition of thymocytes from restoring the immune response. The ability to respond partially recovers in less than 17 days but then is similar to the response of non-pretreated mice given only a single dose of thymocytes.

DISCUSSION

Before entering into a discussion of the effects of the various treatment schedules, it is important to consider the reasons for the lack of specificity noted in these experiments. In some of the groups studied, pretreatment with SRBC led to a significant suppression of the subsequent response to HRBC. It was however, always of lesser magnitude and duration than the effect on the response to the homologous antigen. Three possible explanations for these observations have been considered.

(1) A non-specific immunosuppression, such as reticulo-endothelial blockade, produced by the noxious effects of the injection of large numbers ($2{\cdot}5\times 10^{10}$) of heterologous red cells. This explanation is unlikely as the injection of animals deprived of TD lymphocytes had no effect at all on the ability of thymocytes to restore the response to HRBC. Since the same injections given in the presence of TD cells depressed the HRBC response, even after the addition of more thymocytes, it would appear that the presence of TD cells was a causative factor.

(2) One mechanism by which TD cells could have acted is through antigenic competition (Adler, 1964; Radovich and Talmage, 1967). To test this possibility some non-pretreated animals were given only a single injection of SRBC 4 days prior to the inoculation of HRBC and thymocytes. Their response to HRBC was not impaired. This explanation is also weakened by the observation that antigenic competition does not occur in animals unresponsive to one of the competing antigens (Wust and Hanna, 1966; Liacopoulos, Perramant and Herlem, 1967; Weigle and High, 1967) although an exception has been noted with very closely related antigens (Schechter, 1968). In the experiments reported above the response to HRBC was depressed in animals that made no response to SRBC.

The possibility that SRBC antigen remaining from the pretreatment competed with the HRBC for the new (non-tolerant) thymocytes added 4 days after the termination of the pretreatment may be eliminated because this did not occur in animals deprived of TD cells during treatment. Furthermore, antigenic competition is a relatively short lived phenomenon (<10 days) (Radovich and Talmage, 1967), whereas suppression of the HRBC response was present in some of our experiments for much longer periods of time.

(3) A third possibility is that cross tolerance was produced even though the antibodies made in response to challenge with SRBC are not supposed to cross-react with HRBC (Cunningham, 1966; Radovich and Talmage, 1967). Since it has been reported that *Salmonella* antigens which do not elicit cross-reacting antibodies can induce cross-reacting tolerance, it is possible that tolerance and immunity have different specificities (Austin and Nossal, 1966). Further evidence dissociating the specificity of the immune response from

the specificity of tolerance has recently been reported (Bauminger and Sela, 1969). However, although we confirmed the reported absence of cross-reactions between SRBC and HRBC in the primary response prior to initiating these experiments, we have discovered more recently that hyperimmunization may lead to high titres of cross-reacting antibodies. This observation makes the possibility that we are observing cross-reacting tolerance more feasible.

Drug induced tolerance to SRBC has been noted to result in a diminished response to a wide variety of heterologous erythrocytes (Frisch and Davies, 1966; Dietrich and Dukor, 1967). We have been able to find only one report in the literature of the HRBC response made by mice tolerant to SRBC (Miller and Mitchell, 1968). Although a normal response to HRBC was noted by those authors, the difference in results might be explained by several factors. Our protocol of tolerance induction for example involved considerably more antigen administration which, as noted above, is more likely to affect those cells making cross-reacting antibodies. A similar finding in the induction of tolerance to BSA has recently been reported (Paul, Thorbecke, Siskind and Benacerraf, 1969). Also, the small numbers of thymocytes present in our mice may have allowed some cross-reactions to be seen that might not otherwise be obvious. Furthermore, Mitchell and Miller did not test for tolerance until 3–4 weeks after induction, during which time new cells could have regenerated from the thymus.

There are some reports in the literature that suggest cross-reacting tolerance may exist in TD cell populations. For example, the mitotic response of TD cells to various heterologous red cells including HRBC may be abrogated by multiple injections of SRBC, (Gershon *et al.*, 1968; Davies, 1969). It has also been noted that pretreatment of animal with BSA impaired the ability of their thymocytes to co-operate with normal bone marrow, in the response to HSA (Taylor, 1969).

Whatever the explanation may be, since the cross-reacting suppression was dependent upon the presence of thymocytes, it is most likely that the basis for it was immunological rather than non-specific.

Assuming then that the suppression of the SRBC response produced by prior contact with homologous antigen is a form of tolerance, or at least related to it in some way, what have these experiments shown?

The basic question asked was whether tolerance to SRBC in the mouse was, as the immune response is known to be, thymus dependent. Tolerance was tested in two ways. One was by the ability of mice to respond to a test dose of antigen, given at 4 and 21 days after the termination of tolerance induction. The second was by testing the ability of the cells in the treated animal to interact with an injection of normal thymocytes, also given 4 days after the termination of tolerance induction.

The results showed that the SRBC treatment in the absence of thymocytes could eliminate the small transient MES antibody response that mice without TD cells are capable of making. It could not, however, influence the ability of added thymocytes to restore the MER antibody response.

On the other hand, the same pretreatment with antigen given in the presence of 15×10^6 thymocytes not only made mice unresponsive to further antigen injections, it also temporarily prevented the addition of thymocytes from having a restorative function. Although mice not given thymocytes remained unresponsive to SRBC for longer than 3 weeks, mice given a second injection of thymocytes were able to respond to an immunizing dose of SRBC given 17 days after the first (21 days after termination of tolerance induction).

Thus, the interaction of SRBC and thymocytes had temporarily resulted in an abrogation of the ability of the BMD cells to co-operate with normal thymocytes. It is indeed possible that the BMD cells had been made tolerant. Other workers have noted an inability of thymocytes to break tolerance to SRBC, although they did not show the thymic inoculum used was active in non-tolerant animals (Denman, Vischer and Stastny, 1967).

The loss of tolerance in less than 17 days can most simply be explained by regeneration of new cells from the bone marrow. These new cells, although probably also present in animals without a second inoculation of thymocytes, cannot respond in that case, as the only TD cells present are themselves tolerant.

It seems unlikely that the thymocytes added after pretreatment became tolerant because they were shown to be reactive 17 days after being added.

Before considering the mechanisms by which tolerance might have occurred, there is one other result that should be considered. This is the depressed response, on days 5 and 7, of animals pretreated in the absence of thymocytes and then given thymocytes. This result does not indicate that the thymus dependent response was affected by the pretreatment. If the thymus independent response of non-pretreated animals is added to their response, it then becomes similar to that of the non-pretreated controls given thymocytes. Since it was shown that the thymus independent response was completely abrogated by pretreatment it is possible that only this part of the response was lacking. In other words, those antibody making cells that could produce antibody without assistance from TD cells were tolerant and the addition of thymocytes could not restore their reactivity. The observation that the early depressed response of these animals was related in time to the addition of thymocytes and not to the termination of pretreatment indicates that the recovery noted at day 10 was not due to regeneration of new cells from the bone marrow. Thus, no indication that thymus dependent BMD lymphocytes can be affected by antigen pretreatment in the absence of TD cells was found in these experiments.

Rather it would appear that most thymus-dependent BMD lymphocytes are incapable of reacting to antigen without some form of assistance; they previously have been shown to be incapable of making antibody (Davies *et al.*, 1968) and in this work they have been shown to be incapable of becoming tolerant. They appear to have both capabilities in the presence of TD cells. Thymus independent BMD cells, on the other hand, have both capabilities in the absence of TD cells.

Two alternate mechanisms may be suggested for how the TD cell participates in the production of tolerance.

It may act in the same fashion as it does in the production of immunity. That is it makes some substance (IgX?) (Mitchison, 1968a), which facilitates the interaction of antigen and potential antibody making cell. The antigen concentration at the level of the facilitated cell then determinates whether the cell will produce immunoglobulin or be paralysed. A weakness of this hypothesis is the difficulty in accounting for the facilitation occurring even though the TD cell itself is being made tolerant in the process.

The other suggestion we might offer is that the TD cell not only makes a facilitating substance, but also a 'shut-off' substance (IgY?). Tolerance in the presence of large amounts of antigen could be caused by an excess production of IgY which would shut-off both BMD+TD lymphocytes.

Although we find the second hypothesis attractive because it would be useful in explaining, in a unitary manner, a number of immunological observations, there is no direct evidence to substantiate it.

Whatever the mechanism, the need for co-operation of cells to produce tolerance can explain the difficulties there have been in producing tolerance *in vitro* (Mitchison, 1968b; Dresser and Mitchison, 1968). Mosier (1969) has shown why cellular interactions have made the production of a primary immune response *in vitro* so difficult to achieve until recently (Mishell and Dutton, 1966). It is of some interest that the only reports of tolerance production *in vitro* (with thymus dependent antigens) are under conditions where a primary immune response can also be induced (Diener and Armstrong, 1967; Scott and Waksman, 1969).

Similarly, since tolerance like immunization requires a TD cell response (again, for thymus dependent antigens) the same reason may be used to explain why D polymers which are non-immunogenic, are also incapable of producing tolerance (Collotti and Leskowitz, 1969). If they are unable to stimulate TD cells they are then immunologically inert, even though they can combine *in vitro* with antibodies, produced by immunization with L polymers.

One final point is worth emphasizing. That is the difference between these experiments and those that test co-operation between thymocytes and bone marrow cells (Taylor, 1969). We have not studied bone marrow cells themselves but rather peripheral lymphocytes derived from the bone marrow in the absence of a thymus. Several potential differences exist, between these two cell populations that might result in different results in experiments on tolerance. For example an inability to demonstrate tolerance in bone marrow cells could be explained by rapid regeneration of cells (Taylor, 1969), by an absence of TD cell influence on cells in the marrow, by the need for these cells to pass through another source organ, or by other factors not operative in peripheral tissues. The rapid recovery of the peripheral BMD lymphocytes from tolerance in our experiments might suggest that the cells in the bone marrow of our experimental animals were not tolerant either.

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